

CHARACTERIZATION OF WALL TEICHOIC ACIDS
IN TWO MORPHOLOGICAL FORMS OF
Arthrobacter crystallopoietes

By

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Abstract of Dissertation Presented to the Graduate Council
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The cell wall teichoic acid isolated from two morphological forms of *Arthrobacter crystallopoietes* is characterized. Cell walls purified from spherical (GS) cells contained 13.2 μg of phosphorus per mg of cell wall, while those from rod-shaped (LS) cells contained 21.2 μg phosphorus per mg of cell wall. Trichloroacetic acid extracts of purified walls of both forms were found to contain poly (glycerol phosphate) with hexosamine glycosidically attached. In GS teichoic acid there was 28% as much hexosamine as glycerol phosphate and in LS teichoic acid there was 21% as much. The hexosamine included at least 50% N-acetylated glucosamine and galactosamine in about a 3-to-1 ratio. Evidence is presented which suggests that the hexosamine may exist as trisaccharide side chains. Chain lengths were estimated by the ratio of total phosphate to alkaline phosphatase-labile phosphate. By this method, teichoic acids from GS-grown cells had an average length of 33 glycerol phosphate

units and those from LS-grown cells had an average length of 70 units. The possible significance of these findings as they relate to morphogenesis in *Arttrobacter* is discussed.

INTRODUCTION

Taxonomy

The genus *Arthrobacter* is characterized by cells which can undergo nutritionally-controlled sphere-rod morphogenesis (48). The species *Arthrobacter crystallopoietes* was first described by Ensign and Rittenberg (30) who isolated the organism by enrichment cultures containing 2-pyridone. The brilliant green crystalline pigment produced by this species growing on solid medium containing 2-pyridone was later identified by Kuhn et al. (57) to be a hydrate of the monopotassium salt of 4,5,4',5'-tetrahydroxy-3,3'-diazadiphenquinone-(2,2'). Recently, Kolenbrander and Weinberger (52) found that *A. crystallopoietes* lost the ability to produce pigment from 2-pyridone at a high spontaneous frequency of 0.26% loss per generation. This high spontaneous loss has also been observed in the strain of *A. crystallopoietes* used in this dissertation research. Kolenbrander and Weinberger (52) present good evidence that the loss of a plasmid is correlated with loss of ability to produce pigment. In the present study this presented a problem since the plasmid-less strain seemed to grow faster than the parent strain in glucose-salts media. It was therefore necessary to minimize the number of accumulated mutants by starting each culture for harvest from a single pigment producing colony.

According to Bergey's Manual (48), the name *Arthrobacter crystallopoietes* is a probable subjective synonym of *Arthrobacter globiformis*. The Manual also states that the salient feature distinguishing *A. crystallopoietes* from *A. globiformis* is the ability to utilize 2-pyridone as a sole carbon and energy source and to produce a crystalline pigment from it. It thus seems likely that *A. crystallopoietes* which has lost the plasmid has almost exactly the same phenotype as *A. globiformis*. Since the presence or absence of the plasmid may affect the wall composition by either direct effects (e.g., plasmid genes might modify wall synthesis) or indirect effects (e.g., plasmid presence might affect growth rate which might in turn affect wall synthesis), comparisons between the walls of these bacteria can not be made on the assumption that *A. crystallopoietes* and *A. globiformis* are the same bacteria.

The nutritional control of morphology in *A. crystallopoietes* was first demonstrated by Ensign and Wolfe (31). They showed that exponential growth of spheres could be obtained in a defined medium containing glucose. Exponential growth of rods could be obtained by adding certain morphogenesis-inducing compounds to the defined glucose medium. This idea forms the basis for the methods of obtaining spheres and rods in the following work. The only difference is that the rod-inducing medium contains lactate but no glucose. Since there is a diauxic suppression of glucose catabolism and anabolism in the presence of rod-inducing compounds (54), this difference is minimized.

Control of Morphogenesis

Although the morphology of *Arthrobacter* can be extrinsically controlled by nutritional means, this control is only indirectly related to the unknown intrinsic control by the bacteria. The intrinsic control is more directly related to growth rate which can be controlled independently from the type of nutrition. Luscombe and Gray (60) have done this by growing a strain of *Arthrobacter* under carbon-limiting conditions in a chemostat. They found that, at 25°C, rods were only produced at dilution rates above 0.25 per hour. At rates lower than this, the cells were always spherical. This raises a problem because it means that whenever rod and sphere-shaped arthrobacters are compared, morphology is not the only difference; growth rate also changes and hence could affect many variables. This is a fundamental problem which makes it difficult to establish correlations as causal relationships and it applies to this study. Hamilton et al. (39) have sidestepped this problem by isolating a spherical morphological mutant of *A. crystallopoietes* which is unable to undergo sphere-rod morphogenesis but increases its growth rate in rod-inducing media. The genetic nature of this lesion has not yet been reported.

The intrinsic control of morphogenesis probably consists of a chain of events, some of which are genetic, some enzymatic, and some structural. Certain changes at any level of this chain can probably manifest themselves at the morphological level. Therefore, any one event should not be said to control morphogenesis. Nevertheless, several authors (39,47,53,61,68,69,73) claim that their effect which

correlates with morphology is probably the factor which controls morphogenesis in *A. crystallopoietes*.

It has been shown that *A. crystallopoietes* contains two RNA polymerases both of which have considerable and almost equal synthetic capabilities in *in vitro* studies (47). This correlates with another finding by the same lab (61) that morphogenesis involves differential transcription of the DNA with some transcripts being present all the time and others being present only at certain times during the morphogenetic cycle. Furthermore, St. John and Ensign (73) by using RNA and DNA synthesis inhibitors were able to show that morphogenesis can occur in the absence of DNA replication but RNA synthesis is required for morphogenesis to occur.

Hamilton et al. (39) suggest that the level of cyclic adenosine 3',5'-monophosphate (c-AMP) may be important in regulating morphogenesis. The levels of c-AMP were shown to rise steeply at times just prior to the initiation of shape changes (either sphere-to-rod or rod-to-sphere). This suggested that elevated c-AMP levels may act as the "trigger" which induces the cells to change shape. Their morphological mutant (mentioned above) did not show these changes.

Krulwich et al. (53, 55) have shown that the glycans in the peptidoglycan of spheres are generally shorter than those of rods. This change was also correlated with a change in the activity of a wall-bound N-acetylmuramidase. They implied that morphology, therefore, depends on glycan chain length.

Previc (68) has suggested that morphology in most bacteria may be determined by the presence or absence of extra crossbridges involving free carboxyl groups of diaminopimelic acid (Dpm) and other

tetrafunctional amino acid groupings (e.g., lysylaspartyl crossbridges in some *Lactobacillus* species). In a mutant of *A. crystallopoietes* Previc and Lowell (69) have shown that spherical mutants contain lysine in the penultimate position of tetrapeptides while rod-shaped mutants contain Dpm. Transitional stages during sphere-to-rod morphogenesis show a gradually increasing amount of Dpm present. Thus, morphology in this strain may be dictated by the presence or absence of the extra carboxyl group of Dpm.

Several authors seem to agree that a rod shaped morphology requires a more rigid peptidoglycan than a spherical morphology (37,55,68). Ward and Claus (84) have found that for *A. crystallopoietes* the rod peptidoglycan layer is thinner than the sphere peptidoglycan. If the rod peptidoglycan is more rigid than the sphere peptidoglycan, then this difference must be due to changes in the peptidoglycan structure rather than simply a thickening of the wall.

It should now be apparent that there are changes during morphogenesis at each step in the chain of events which regulates morphology. What is still not apparent is how all these changes fit together to produce morphogenesis. Of course, some of the changes may not be related to morphogenesis at all but only reflect altered growth rates. So, the question of how morphogenesis occurs is still unanswered.

Justification for This Study

The original intent of this author was to determine how peptidoglycan might help to maintain the different shapes of *A. crystallopoietes*. To accomplish this the composition of the wall was examined for changes

which might correlate with morphology. During the beginning of the quantitative analyses of the components of the wall, there arose certain evidence that a wall teichoic acid might be present. The determination of its qualitative and quantitative structure has become the primary topic of this dissertation. Although this seems far removed from the question of morphogenesis, there may be an intimate association between the two. These possibilities are examined in the Discussion.

Teichoic Acid Background

Teichoic acids are molecules which occur in nearly all gram-positive bacteria (3). These molecules are grouped into two categories depending on their cellular location and their structure; lipoteichoic acids are found associated with the cell membrane and wall teichoic acids are associated with the peptidoglycan (4).

Lipoteichoic acids are found in most gram-positive bacteria. These molecules are all of the same structural type, i.e., they consist of a linear backbone of poly (glycerol phosphate) which is linked by phosphodiester bonds involving C-1 and C-3 of adjacent glycerol phosphates (3). Diversity of lipoteichoic acid structure is introduced by various carbohydrate side groups (see Table 1) which are attached at the C-2 hydroxyl group. D-Alanine is usually found as an ester linked either to the C-2 hydroxyl of glycerol phosphate or to glycosyl hydroxyl groups. It is though that all lipoteichoic acids are covalently attached to glycolipid in the cell membrane (51).

Wall teichoic acids are more structurally diverse than lipoteichoic acids. The classical wall teichoic acids are polymers of glycerol or ribitol phosphate. Both types can have various carbohydrate side groups (see Table 1) and/or D-alanine. These polymers are thought to be covalently attached to the peptidoglycan in the cell wall. Other acidic polymers have been found in the walls of gram-positive bacteria which resemble the classical teichoic acids. Polymers of glycerol phosphate and one or more sugars have been found in a number of cases and polymers of sugar phosphates are also known. Both types of polymers are similar to teichoic acids in that they convey a net negative charge to the outer surface of the cell and, therefore, may have functions similar to the teichoic acids (51).

TABLE 1

Carbohydrate constituents of representative teichoic acids.^a

Location	Polyol	Carbohydrate ^b	Occurrence
Membrane	Glycerol	Glc Glc- α -1 $\rightarrow$ 2-Glc Glc- β -1 $\rightarrow$ 6-Glc Gal, Gal- α -1 $\rightarrow$ 2-Glc	<i>L. helveticus</i> NCIB 7220 <i>S. faecalis</i> 39 <i>S. aureus</i> H <i>L. fermenti</i> NCTC 6991
Wall	Glycerol	Glc	<i>B. subtilis</i> Marburg <i>L. buchneri</i> NCIB 8007 <i>S. epidermidis</i> T1 <i>S. epidermidis</i> (albus) NCTC 7944
	Ribitol	Glc GlcNAc GalNAc	<i>B. subtilis</i> W-23 <i>L. Plantarum</i> (arabinosus) 17-5 <i>S. aureus</i> Copenhagen <i>S. aureus</i> phage type 187

(a) From K.W. Knox and A.J. Wicken (51).

(b) Glc, D-glucose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine.

MATERIALS AND METHODS

Organism

Arthrobacter crystallopoietes ATCC 15481 was obtained from the American Type Culture Collection. In order to maintain the presence of the plasmid which bears the gene(s) responsible for green crystal production from 2-pyridone, stock cultures were frequently streaked on 2-pyridone-containing plates (30) and pigmented colonies were picked for further subculturing. All inocula for cultures to be harvested were also checked in this manner.

Media

Defined media contained, per liter: 1.73 g K_2HPO_4 , 2.33 g KH_2PO_4 , 1.00 g $(NH_4)_2SO_4$, 5.0 g D-glucose (GS) or sodium lactate (LS), 0.5 g $MgSO_4$, and 10.0 ml of a trace salts solution. The trace salts solution contained, per liter: 1.5 g nitriloacetic acid, 0.5 g $MnSO_4$, 1.0 g NaCl; $FeCl_2$, $CaCl_2$, $CoCl_2$, and $ZnSO_4$, 0.1 g each, and $CuSO_4$, $AlK(SO_4)_2$, H_3BO_3 , and $(NH_4)_6Mo_7O_{24}$, 0.01 g each. The trace salts solution was adjusted to pH 7.0 with 1.76 g NaOH. The glucose, $MgSO_4$, and the trace salts solutions were each autoclaved separately (31).

A complex pigmentation medium containing 0.2% 2-pyridone, 0.05% yeast extract, and inorganic salts was prepared according to Ensign and Rittenberg (30).

Growth

All cultures were agitated at 30°C in a New Brunswick Gyrotory shaker-incubator. Optical density (O.D.) was followed at 450 nm on a Beckman DU-2 spectrophotometer with a 1.0 cm light path. Optical densities were maintained between 0.05 and 1.0 O.D. units by periodic transfers to fresh media.

Cell Wall Isolation

Exponential cultures were passed through coiled copper tubing which was submersed in an ice-water bath. The cooled effluent was directed into a De Laval Gyro Tester Laboratory Centrifuge with the bowl prechilled to 4°C. The time for culture liquid to pass through the cooling coils and out the centrifuge spout was measured using enough nigrosin dye added to a deionized water run to serve as a visual marker. The temperature and O.D. at 450 nm of the final effluent were also measured during bacterial harvests. From these measurements it was concluded that cultures were cooled from 30°C to 8-10°C in approximately one minute and that 95% or more of the culture's bacterial mass was removed from the effluent as determined by O.D. measurement. All subsequent handling of bacterial samples was at no higher than 4°C unless otherwise specified. Whole cell pellets were washed twice by resuspension in deionized water and centrifugation at 13,000 x g for 30 min in a Servall GSA rotor. Bacteria were suspended in deionized water at a concentration no greater than 50 mg dry wt/ml and broken in a Braun homogenizer at 4,000 rpm for 4 minutes using 0.11 mm

diameter glass beads. Tributyl phosphate (0.5% v/v) was used to reduce foaming. Glass beads were removed first by filtration on fritted glass filters and then by centrifugation at $160 \times g$ for 30 min. Crude cell walls were then pelleted from the supernatant at $27,000 \times g$ for one hour.

Purification of Cell Wall

The method of Braun and Sieglin (14) for wall purification was modified by an additional sodium dodecyl sulfate (SDS) extraction before treatment with pronase (69). Crude wall pellets were suspended in 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 7.4 (at least 20 ml/g of whole cell dry wt), and then pelleted at $27,000 \times g$, washed and resuspended in deionized water. Suspensions were added dropwise to stirred, boiling 4% SDS (at least 15 ml/g of whole cell dry wt). These mixtures were allowed to cool to room temperature with stirring overnight. These mixtures were then centrifuged at $27,000 \times g$ for one hour. Pellets were resuspended in deionized water and reextracted with boiling 4% SDS. These pellets were washed with deionized water three times and resuspended in 0.05 M Tris, pH 7.4 (at least 5 ml/g of whole cell dry wt). Pronase was added to a final concentration of 100 $\mu\text{g/ml}$ and the mixtures were digested at 37°C for 16 hours. The walls were centrifuged for one hour at $37,000 \times g$. The resultant pellets were reextracted by boiling SDS as above and rewashed three times with deionized water. The final pellets were lyophilized for dry weight determinations.

Teichoic Acid Isolation

To release teichoic acid (6), purified walls were extracted with 10% (w/v) trichloroacetic acid (TCA) at 4°C for 24 hours. Walls were pelleted from this mixture at 27,000 x g for one hour and the pellets were reextracted with fresh 10% TCA at 4°C for 24 hours. Again fresh cold TCA was exchanged for supernatant TCA and the extraction was continued for 38 hours. Crude teichoic acids were collected either by precipitation from the TCA with absolute ethanol (5 volumes) at -20°C and subsequent centrifugation at 13,000 x g for 30 minutes, or by removing the TCA by ether extraction to pH 4 and subsequent lyophilization.

Analysis of Purified Walls

Purified walls (0.4 mg/ml) were hydrolyzed in 4 N HCl at 105°C for 11 hours. The excess acid was then removed by evaporation, followed by three cycles of deionized water additions and reevaporations. The residues were dissolved in 0.01 N HCl (1 mg wall/ml acid) and then filtered through 0.45 µm Millipore filters. Unsolubilized material was present in negligible amounts. The filtrates were analysed for amino acids and amino sugars on a JEOLCO Automated Amino Acid Analyzer (76).

Paper Chromatography

Whatman papers no. 1, no. 4, and no. 3 MM were soaked for at least 30 minutes in a solution which was 0.1 N in acetic acid and 0.1 M in EDTA. This solution was then washed out by suspending the papers in deionized water and then removing excess water by decantation. At least ten sequential water washes of increasing duration were performed, with a final wash of at least one hour. Washed papers were allowed to dry in horizontal stacks at 30°C. Papers to be eluted parallel to the grain of the paper were cut to 23 cm x 57 cm with the grain running parallel to the long axis of the paper. Papers to be eluted perpendicular to the grain were cut to 23 cm x 46 cm with the grain running perpendicular to the long axis. Samples were spotted 7 cm from the top of the papers by repeated application of approximately 0.25 µl each time, then drying by hot air. The papers were mountain-folded along a line at 6 cm and valley-folded along a line at 3 cm from the top of the papers. This folding allowed the papers to be hung from a glass trough in a pyrex tank (30.5 cm x 30.5 cm x 61 cm). The papers were allowed to equilibrate with the vapor phase of the eluting solvent in a glass chromatography tank for at least 2 hours before the eluting solvent was added for the beginning of elution. All chromatograms were eluted at ambient temperature, typically ranging from 22°-27°C. The solvent systems for elution used were: (A) n-propanol-ammonia (28-30%)-water (6:3:1) (40) and (B) ethyl acetate-pyridine-water (10:4:3) (36).

Analytical Procedures

Phosphate Assays

Total phosphate was assayed by the ascorbic acid-molybdate method developed by Chen et al. (23). The ashing method of Lowry et al. (58) was used for convenience in handling large numbers of samples.

Inorganic phosphate was measured by the Chen method as modified by Ames (1). KH_2PO_4 (anhydrous analytical reagent, Mallinckrodt) was used as a standard in both total and inorganic phosphate methods. Chromic acid-washed tubes were used for all phosphate assays.

Amino Sugar Assays

N-Acetyl amino sugars were measured by the borate-p-dimethyl-amino-benzaldehyde (DMAB) method of Reissig et al. (70).

N-Acetylglucosamine (Sigma) was used as the standard for this assay. Amino sugars were assayed for N-acetylation with acetic anhydride by a method similar to the N-acetyl amino sugar assay as described by Ghuyssen et al. (35). This method primarily detects amino sugars which are free in the C-1 position but it also detects amino sugars which are covalently bound at that position. The extinction coefficient for the latter reaction, however, is more than thirty fold smaller than that for the former reaction. D-Glucosamine-HCl (K+K Laboratories, Inc.) was used as a standard for this assay.

Total amino sugars could be detected if an acid hydrolysis (2 N HCl, 100°C, 3 hours) followed by neutralization preceded the N-acetylation step of the amino sugar assay. The same standard was used as for the amino sugar assay.

The minimum amount of N-acetylation of amino sugar was estimated by a method suggested by Ellwood et al. (29). This method involves a mild acid hydrolysis (0.1 N H_2SO_4 , 100°C, 30 min in a sealed ampoule) which breaks the glycosidic linkage while leaving most of the N-acetyl groups intact. After hydrolysis, a slight excess (10%) of the amount of BaCO_3 necessary for neutralization was added. The resulting BaSO_4 - BaCO_3 mixture was removed by low speed centrifugation. N-Acetylhexosamines and total amino sugars were then determined for the neutralized samples.

Carbohydrate Assay

Simple sugars, oligosaccharides, and polysaccharides with either free or potentially free reducing groups were determined by the phenol- H_2SO_4 method of Dubois et al. (28). D-Glucose (analytical reagent, Mallinckrodt) was used as a standard.

Protein Assay

Protein was measured by the Lowry method (59) which involves first a reaction of protein with Cu^{+2} under alkaline conditions and then reduction of a phosphomolybdate-phosphotungstate reagent by the copper-treated protein. Bovine serum albumin (Sigma) was used as a standard.

Enzymatic Determination of Glycerol

Free glycerol was determined by using the Glycerol Stat-Pack (Calbiochem). This assay employs the following reaction sequence:

- (1) glycerol + adenosine triphosphate (ATP) glycerol kinase →
α-glycerophosphate + adenosine diphosphate (ADP)
- (2) ADP + phosphoenolpyruvate pyruvate kinase → pyruvate + ATP
- (3) pyruvate + nicotinamide adenine dinucleotide (reduced) (NADH) lactate dehydrogenase → lactate + nicotinamide adenine
dinucleotide (oxidized)

The disappearance of NADH was followed spectrophotometrically at a wavelength of 340 nm using the same instrument used for O.D. readings. Glycerol (analytical reagent, Mallinckrodt) was used as a standard.

Amino Acid and Amino Sugar Analysis

Crude teichoic acid samples (GS, 5.0 mg; LS, 5.6 mg) were each hydrolysed in 1.0 ml of 2N HCl at 100°C for 3 hours. The hydrolysates were vacuum dried over NaOH pellets and then redissolved in 1.0 ml of deionized water. Portions of these (200 µl each) were diluted in 0.01 N HCl to 2.2 ml (final concentrations: GS, 0.45 mg/ml; LS, 0.51 mg/ml). These samples were subjected to amino acid and amino sugar analysis in the same way as described for purified wall hydrolysates.

Teichoic Acid Purification

Crude teichoic acids were purified on approximately 9 g of DEAE-Sephadex A-50. One cm of Sephadex G-25 coarse gel was used at the bottom of the column as bed protection. The DEAE-Sephadex was swollen and loaded in 0.1 M NaCl. The bed diameter was 2.5 cm and the bed length varied depending on ionic strength (35.5 cm at 0.1 M NaCl and 24.0 cm at 1.0 M NaCl). The bed was then washed with 500 ml of 0.1 M NaCl.

Teichoic acids were eluted by an increasing gradient of NaCl. For the LS preparation, 200 ml of a 0.1 M - 0.5 M gradient was followed by a 200 ml gradient from 0.5 M to 1.0 M NaCl. A total of 160 fractions of approximately 2.1 ml each was collected. For the GS preparation, 500 ml of a 0.1 M - 1.0 M NaCl gradient was used of elution. A total of 95 fractions of approximately 5.3 ml each was collected. The fraction numbers for the GS elution profile in the Results section have been normalized with the LS profile using equivalent salt concentrations.

Gel Filtration

Gel filtration was carried out on Sephadex G-100. Purified teichoic acids (GS, 42 mg; LS, 6 mg) were applied to a column bed of dimensions: 2.5 cm x 31 cm. Total bed volume was 160 ml. The void volume was determined to be 54 ml by using Blue Dextran 2000 (Pharmacia, Average molecular weight, 2×10^6). A Blue Dextran sample (1 ml of a 0.2% solution) was applied to a column and the effluent was collected in

approximately 3 ml fractions. These fractions were monitored for O.D. at 260 nm as a measure of Blue Dextran. The number of the fraction with the highest O.D. was multiplied by the fraction volume to obtain the void volume. Eluant was deionized water. A constant pressure head of 31 cm was maintained during loading and running. A Gilson automatic fraction collector was adjusted to collect 95 drop (approximately 3 ml) fractions.

Results were graphed in terms of μ moles of total phosphate vs.

K. K is defined as:

$$K = \frac{V_e - V_o}{V_t - V_o}$$

where: V_o = void volume

V_t = total bed volume

V_e = elution volume

Chain Length Determination

In two separate determinations, about 1 mg and 0.5 mg of each type (GS and LS) of purified teichoic acid was diluted from concentrated solutions to 125 μ l with deionized water. Then, 125 μ l of 0.04 M $(\text{NH}_4)_2\text{CO}_3$ and 10 μ l (0.6 mg or 7.2 units) of *Escherichia coli* alkaline phosphatase (60 mg/ml, 12 units/mg, Worthington Biochemical Corporation, Code: BAPSF - bacterial alkaline phosphatase salt fraction) were added. One unit is defined as that activity liberating one μ mole of p-nitrophenol per minute at pH 8.0 and 25°C. The mixture was incubated in a slowly shaking 37°C water bath for 18 hr. The samples were then analyzed for inorganic and total phosphate content. The ratio of total to inorganic phosphate content was taken as an approximation of chain length.

Determination of Molar Ratios of Glycerol and Phosphate
in Purified Teichoic Acids

Purified teichoic acid samples were hydrolyzed in 2 N HCl at 100°C for 4 hours. Acid was removed by vacuum evaporation or lyophilization. The samples were then digested with alkaline phosphatase by the same general method described under chain length determination. The digested samples were then analyzed for free glycerol, inorganic and total phosphorus. Because of phosphorus present in the enzyme preparation, it was also necessary to run enzyme blanks with no added sample and to subtract out the blank values from the sample values. This method was tested on a known concentration of α -glyceromonomophosphate which produced a molar ratio of glycerol to phosphate of 0.997.

Characterization of Alkaline Hydrolysate

A purified teichoic acid sample (GS, 32.6 μ moles total phosphate) was hydrolyzed in 1.45 ml of 1 M NaOH at 100°C for 3 hours. The hydrolysate was neutralized with 300 μ l of 4 N HCl. The final pH was close to pH 7.0 as judged by pH paper. The neutralized sample was diluted with deionized water to 14.5 ml to attain a salt concentration of 0.1 M NaCl.

The diluted sample was loaded on the same DEAE-Sephadex A-50 column used for the teichoic acid purification. In preparation for this run, the column had been previously washed with 500 ml of 1 M NaCl. The washed column was slowly reswollen with a 1.0 M - 0.1 M

NaCl gradient of 500 ml. Finally, the column was washed with another 500 ml of 0.1 M NaCl for final equilibration. Once loaded, the sample was washed with 500 ml of 0.1 M NaCl. The wash effluent was collected and lyophilized. The adherent portion of the sample was eluted from the column with 500 ml of a 0.1 M - 1.0 M NaCl gradient. Fractions (100, approximately 4 ml each) were collected. These fractions were assayed for total phosphate and total amino sugars.

The lyophilized wash sample was then filtered on a Sephadex G-50 column (diameter 1.6 cm, height 62 cm, 124 ml bed volume, 56 ml void volume) with deionized water in order to resolve low-molecular weight, non-anionic fragments. Fractions (65 of 3 ml each) were collected and analyzed for total and inorganic phosphate and total amino sugars. All phosphate-containing fractions were then pooled and lyophilized. This material (approximately 2.3 g) was assumed to be mostly NaCl and was diluted with deionized water to 0.1 M NaCl on that basis.

This diluted sample was applied to a CM-Sephadex C-50 column (approximately 1.9 g of gel; column with following dimensions: diameter, 1.5 cm, length, 30 cm) to further resolve cationic molecules. The column bed was then washed with 50 ml of 0.1 M NaCl. After washing, the remaining sample was eluted with 250 ml of a 0.1 M - 1.0 M NaCl gradient, and 46 fractions (5.4 ml each) were collected. These fractions were analyzed for total phosphorus and total amino sugars. The two peaks (two fractions/peak) containing significant amounts of hexosamine were each combined and lyophilized. These lyophilized samples were triturated in the presence of absolute

ethanol (10). The ethanol fractions were then evaporated to dryness and the residues were triturated again with absolute ethanol (1 ml). The ethanol-soluble portions were again evaporated to dryness. The residues were each dissolved in 450 μ l of deionized water and then analyzed for free glycerol.

RESULTS

Growth of *Arthrobacter crystallovoietes*

ATCC 15481

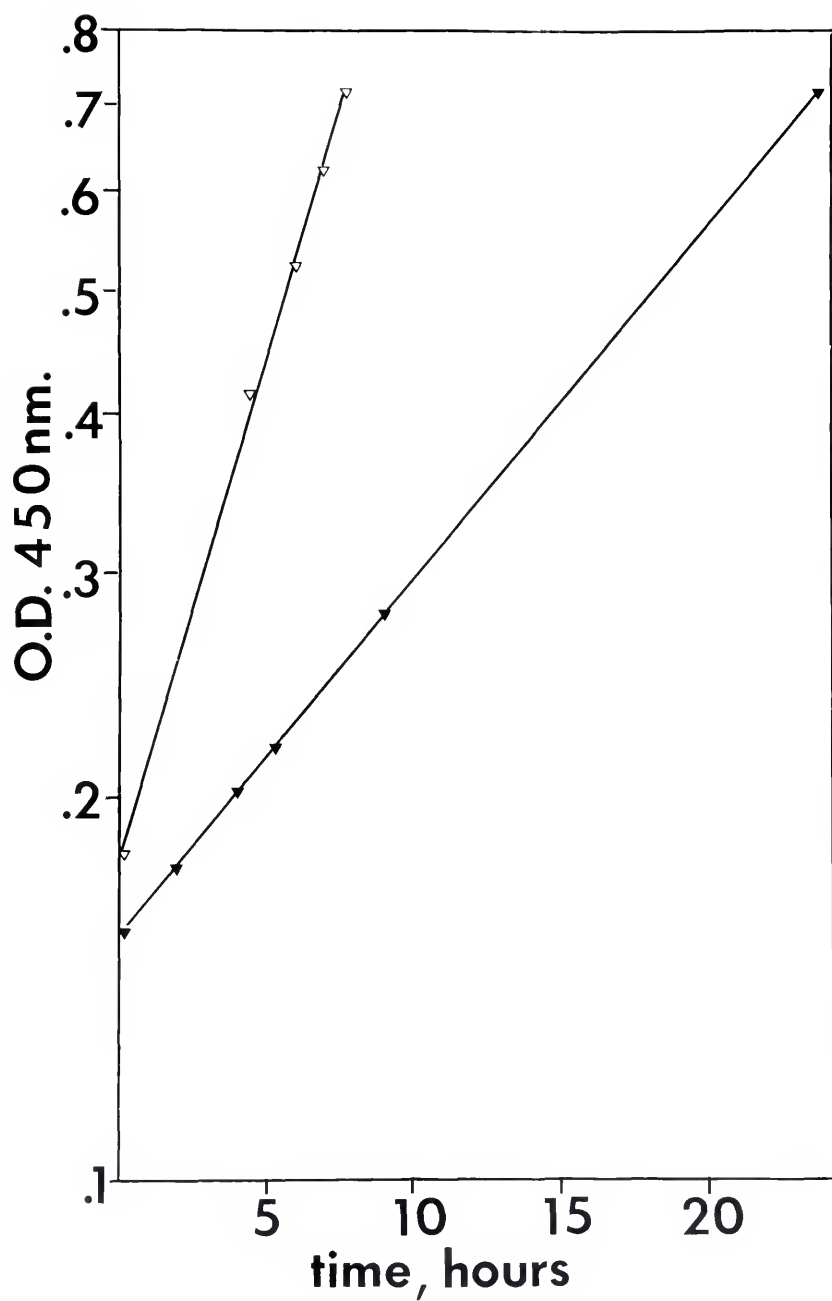
Typical growth curves are shown in Figure 1. Generation times were: GS, 10.8 hr $\pm$ 0.5 hr; LS, 3.5 hr $\pm$ 0.2 hr.

Amino Acid and Amino Sugar Analysis of Purified Wall Hydrolysates

It was observed that cell walls purified according to the protocol in Materials and Methods usually contained certain non-peptidoglycan amino acids. The following experiment was an attempt to remove these amino acids using proteolytic enzymes.

Cell walls were isolated from lyophilized cells (GS or LS, 300 mg each) and were purified as described in Material and Methods with one modification. Just before the pronase digestion, each sample was divided into four equivalent portions. One portion of each was not treated with any enzyme and served as the control; the second portions were treated with pronase (500 μ g/ml); the third portions were treated with trypsin (100 μ g/ml); and the fourth portions were treated with α -chymotrypsin (100 μ g/ml). All digestions were carried out in 0.05 M Tris, pH 7.4 at 37°C for 16 hours. Following the enzymatic digestions, each sample was treated as described in Materials and Methods for the rest of the wall purification and

Figure 1. Growth of *Artinrobacter crystallopietes*. GS (7), LS (7).



amino acid analysis. Peak areas on chromatograms were computed by a JOELCO integrater. Relative concentrations were then computed using known areas of standard amino acids and sugars. These relative concentrations were then divided by the relative concentrations for the glutamic acid peak on each chromatogram. The values for these ratios are given in Table 2. These analyses indicate that the normal peptidoglycan amino acids are present in the expected ratios. They also show that trypsin is the most effective enzyme in removing the trace amino acids.

Chemical analysis of purified cell wall hydrolysates also shows the presence of significant amounts of phosphorus (GS, 18.2 $\mu\text{g P/mg}$ wall; LS, 21.2 $\mu\text{g P/mg}$ wall). Since Krulwich and Ensign (55) had reported phosphorus in their cell wall preparations of *A. crystallopoietes* and had found that all of it could be removed by hot TCA extraction, a similar experiment was carried out with the cell walls prepared in this laboratory.

TCA Extraction of Purified Walls

Purified cell walls (GS, 19 mg; LS, 18 mg) were suspended in 10 ml of cold 10% TCA and were stirred with a magnetic stirring bar at 4°C. At various times, 1.0 ml portions were removed and extracted three times with petroleum ether (B.P. 30°C) to remove the TCA. The sample was then centrifuged at 27,000 x g for a half hour.

Total phosphate was determined for both the soluble and insoluble fractions as shown in Figure 2. Phosphate was extracted by TCA with

TABLE 2

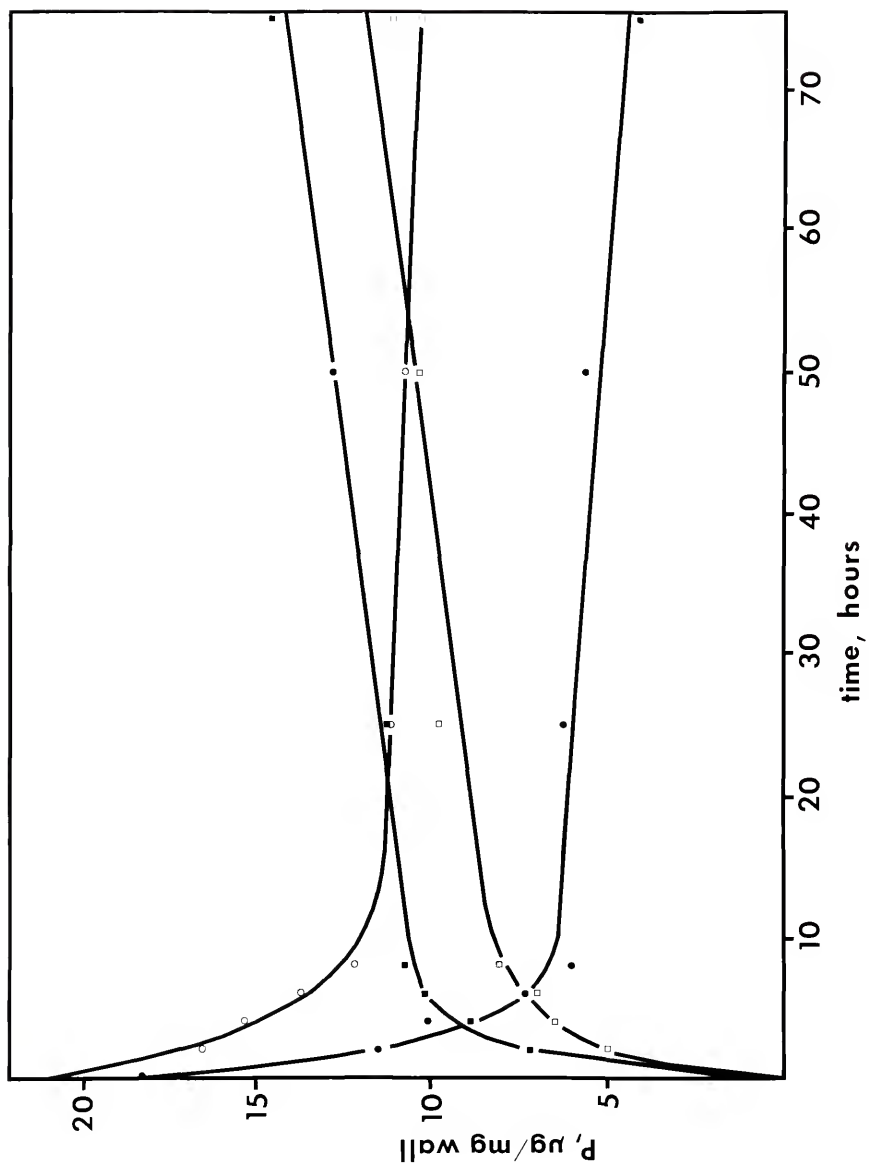
Molar ratios of amino acids and amino sugars in walls digested with various enzymes.^a

<u>Treatment</u>	<u>Control</u>		<u>Pronase</u>		<u>Trypsin</u>		<u>α-Chymotrypsin</u>	
	GS	LS	GS	LS	GS	LS	GS	LS
GlcNH ₂ ^b	1.42	1.53	1.65	1.48	1.59	1.58	1.58	1.44
Lys	0.88	0.94	1.00	0.92	0.94	0.95	0.96	0.86
Asp	0.06	0.04	0.04	0.03	ND	0.01	0.03	0.03
Thr	0.03	0.03	0.03	0.02	ND	T	0.04	0.02
Mur	0.45	0.47	0.45	0.44	0.44	0.46	0.48	0.48
Glu	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Gly	0.04	0.04	0.03	0.02	ND	0.01	0.03	0.02
Ala	2.61	2.64	2.61	2.68	2.75	2.68	2.68	2.68
Val	0.03	0.03	0.02	0.01	ND	ND	0.02	0.02
GlcNH ₂ ^c	1.29	1.28	1.32	1.30	1.33	1.40	1.33	1.41
GalNH ₂ ^d	+	+	+	+	+	+	+	+

- (a) Molar ratios are relative to glutamic acid. ND - not detectable, T - trace, + - present but amount not determined.
- (b) Glucosamine calculated from short column data from amino acid analyzer.
- (c) Glucosamine calculated from long column data.
- (d) GalNH₂ - galactosamine.

Figure 2. TCA Extraction of Purified Walls

Purified cell walls were extracted with 10% TCA at 4°C. At various times, samples were removed, ether extracted, centrifuged and the pellet and supernatant analysed for total phosphate. GS supernatant (■), LS supernatant (□), GS pellet (●), and LS pellet (○).



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time as indicated by the increase in TCA-soluble phosphate. This increase was paralleled by an equivalent decrease in wall associated phosphate as indicated by the TCA-insoluble curves.

Recoveries of Purified Wall and TCA Extractable Material
(Crude Teichoic Acid)

Walls purified according to the scheme outlined in Material and Methods yielded 12-14% wall dry weight to cell dry weight for GS cells and 13-15% for LS cells. In each individual case the LS cells yielded slightly more wall (6-10% more) on a percentage basis than the GS cells.

Recoveries of TCA-extractable material varied depending on whether ethanol precipitation or ether extraction was used to remove TCA from the samples. When ethanol precipitation was used, TCA extractable material recoveries (as crude TA dry weight percentage of purified wall dry weight) were: GS, 21%; LS, 18%. When ether extraction was used the recoveries were much higher: GS, 38% and LS, 28%.

Characterization of Acid-Hydrolyzed TCA Extracts by Paper
Chromatography

To determine the nature of this phosphorus containing compound, acid hydrolyses were performed. The conditions used have been reported to degrade teichoic acids of either polyglycerophosphate or polyribitol phosphate and substituted with either amino acids or carbohydrate (3).

TCA extracts from two separate preparations were hydrolyzed in 2 N HCl at 100°C for 3 hours. The residues were dried *in vacuo* over NaOH and then redissolved in deionized water. Approximately 25 µg of each sample was spotted on each of two washed Whatman #4 papers along with standards. The papers were then run with solvent system A perpendicular to the grain. One paper was run ascending for 32 hours. The other paper was run descending for 5 hours. Both papers were air dried. Phosphoric acid esters were detected by the acid-molybdate spray (40). The results of these chromatograms are given in Table 3. The unknown spots had mobilities characteristic of 1,2-diphosphoglycerol.

Approximately 50 µg of hydrolyzed TCA extract were run under descending conditions in solvent system A but, in this case, α-glycols were detected with periodate-Schiff spray reagent (9). The results are given in Table 4. By detecting α-glycols the acid hydrolysate was shown to contain glycerol and not ribitol.

The acid hydrolysates of teichoic acid were also examined for sugar content. About 50 µg of each sample was spotted on washed Whatman #1 paper along with appropriate standards. The chromatogram was run with an ascending front of solvent system B for 18 hours. After air drying, sugars were detected by the silver nitrate-NaOH spray (19,32). The results are shown in Table 5. The unknowns in this case show mobilities characteristic of glucose and probably glucosamine.

To confirm the identity of glucose in the unknowns, glucose was added to each unknown as an internal standard on similar chromatograms. The results are shown in Table 6. These results tend to confirm the presence of glucose in acid-hydrolyzed crude teichoic acids.

TABLE 3

Paper chromatography of acid-hydrolyzed TCA extracts.^a

I. Phosphoric acid esters.

Standards	<u>R_f</u>	
	Ascending	Descending
1,2 diphosphoglycerol (50 nmoles)	0.14 ^b 0.09 ^c	0.28 ^b 0.16 ^c
α-glycerophosphate (100 nmoles)	0.24	0.39
β-glycerophosphate (100 nmoles)	0.31	0.39
hydrolyzed cardiolipin ^d (50 nmoles)	— ^e	0.35 ^c 0.12 ^b
<u>Samples</u>		
GSTA (25 μg)	0.13 0.09	0.11
LSTA (25 μg)	0.14 0.10	0.14

(a) Elution was with solvent system A and spots were detected with the acid-molybdate spray reagent for phosphoric acid esters.

(b) faint blue spot

(c) dark blue spot

(d) Hydrolysed cardiolipin was used for a 1,3-diphosphoglycerol standard.

(e) not run

TABLE 4

Paper chromatography of acid-hydrolyzed TCA extracts.^a
 II. α -Glycols

Standards	R _f
α -glycerophosphate (600 nmoles)	0.42
hydrolyzed ribitol (65 nmoles)	0.65
glycerol (1 μ mole)	0.75
<u>Samples</u>	
GSTA (50 μ g)	0.73 ^b
LSTA (50 μ g)	0.74 ^b

- (a) Elution was descending with solvent system A and spots were detected using the periodate-Schiff spray reagent for α -glycols.
- (b) Also had yellow spots at the origin which were the only spots to develop color slowly.

TABLE 5

Paper chromatography of acid-hydrolyzed TCA extracts.^a
 III. Sugars (external standards).

Standards	R_{glucose}^b
Glucose (5 μg)	1.00
Galactose (5 μg)	0.88
Glucosamine (5 μg)	0.39
Galactosamine (5 μg)	0.30
<u>Samples</u>	
GSTA (50 μg)	1.01 0 - 0.53 ^c
LSTA (50 μg)	1.10 0 - 0.53 ^c

- (a) Elution was ascending with solvent system B and spots were detected by the silver nitrate-NaOH spray reagent for sugars.
- (b) $R_{\text{glucose}} = \text{cm sample spot migrated/cm D-glucose migrated}$.
- (d) Tailing which included darker areas at $R_{\text{glucose}} = 0.42$ and 0.32 . These darker areas were also reactive with a DMAB spray reagent (11).

TABLE 6

Paper chromatography of acid-hydrolyzed TCA extracts.^a
IV. Sugars (internal standards)

Standard	^b R_{glucose}
Glucose	1.00
<u>Samples</u>	
GSTA + glucose ^c	0.94 0 - 0.42 ^d
LSTA + glucose ^c	0.98 0 - 0.43 ^d

- (a) Elution was ascending with solvent system B and spots were detected by the silver nitrate-NaOH spray reagent for sugars.
- (b) R_{glucose} = cm sample spot migrated/cm D-glucose migrated
- (c) To 50 μg of each sample spot, 5 μg of D-glucose was added as an internal standard.
- (d) Tailing which included darker areas similar to those in Table 5.

Amino Acid and Amino Sugar Analysis
of Crude Teichoic Acid

When crude teichoic acid samples were subjected to amino acid and amino sugar analysis, the only major detectable peaks corresponded to glucosamine and galactosamine. In both types of teichoic acid samples (GS and LS), the galactosamine was a minor but not negligible amount of the total hexosamine (GS, 18%; LS, 16%). There were traces of alanine in both samples, but there was too little to be quantified by the present method.

These data raised the question of whether the carbohydrate found in acid hydrolysates was covalently attached to the presumed teichoic acid or not. Since the carbohydrate material consisted of neutral or cationic sugars, it was decided that the teichoic acid could be purified by DEAE chromatography. By allowing the anionic teichoic acid to stick to the DEAE groups, the carbohydrate, if not covalently linked, could be washed through the column. Then the teichoic acid could be recovered by eluting with a NaCl gradient.

Crude Teichoic Acid Purification on DEAE-Sephadex

Crude teichoic acids (GS, 142 mg; LS, 100 mg) were loaded on DEAE-Sephadex and washed with 0.1 M NaCl. A gradient of NaCl was used for elution (for specific details see Materials and Methods). The washings and eluate were examined for the presence of phosphorus, reducing groups, and N-acetylhexosamine. The washings showed negligible amounts of either acid-molybdate or borate-DMAB reactive

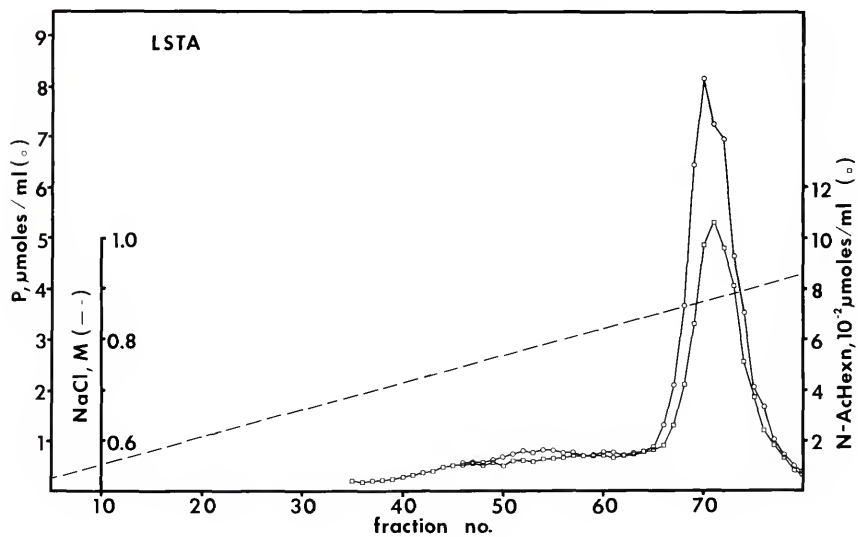
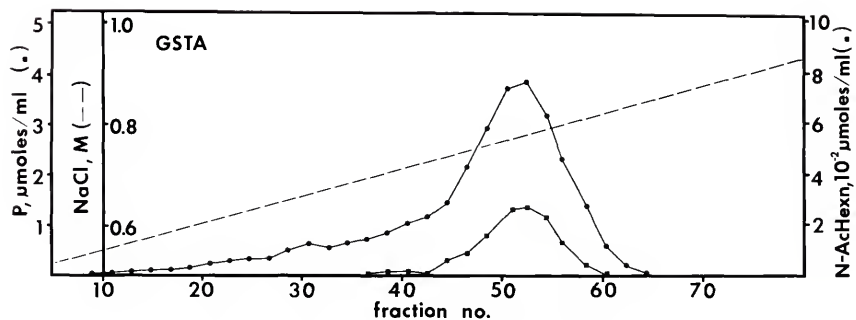
material. There was, however, some phenol-H₂SO₄ reactive material present. This amounted to about 5 μ moles of reducing groups for both GS and LS. The eluate profiles are shown in Figure 3. No reducing groups were found in these fractions. The major portion of the phosphate-hexosamine peaks eluted at NaCl concentrations of 0.73 M - 0.82 M for the GS preparations and 0.85 M - 0.91 M for the LS preparation. Both preparations had a smaller amount of material which eluted at much lower NaCl concentrations. Fractions 66-80 for LSTA and 43-60 for GSTA were pooled, dialyzed, and lyophilized. Dry weight recoveries were: GS-45.1 mg (32% of total material loaded on column) and LS-6.6 mg (7% of total material). Total phosphate recoveries were GS-70% (109 μ moles recovered from 156 μ moles applied to column) and LS-77% (109 μ moles recovered from 140 μ moles applied).

These purified teichoic acids were analyzed for total amino sugars. This analysis showed that there were 28% as many amino sugar residues as phosphate residues for the GS teichoic acid and 21% as many for the LS teichoic acid. By using a mild acid hydrolysis, it was possible to release the amino sugars with some of the N-acetyl groups originally present still intact. This approach yielded 47% (GS) and 54% (LS) of the total amino sugar as N-acetyl hexosamine.

To further characterize the teichoic acids, they were analyzed by gel filtration.

Figure 3. DEAE-Sephadex Chromatography of Crude Teichoic Acid

Crude teichoic acids were applied to 9 g of DEAE Sephadex. The column was washed with 500 ml of 0.1 M NaCl. A salt gradient from 0.1 M to 1.0 M NaCl was used for elution. Fractions were assayed for total phosphate and N-acetylhexosamine.



Purified Teichoic Acid Characterization
on Sephadex G-100

Purified teichoic acids (GS-42 mg, LS-6 mg) were run on Sephadex G-100 Fine in deionized water. The results of these runs are shown in Figure 4. K values for peak fractions were: 0.20 for LSTA and 0.71 and 0.69 for two separate isolations of GSTA (0.69 profile not shown).

These results suggested that the molecular weights of the teichoic acids might be relatively high and that the LS teichoic acid was substantially larger than the GS teichoic acid. To test this hypothesis, the average chain length was estimated.

Chain Length Determination

Purified teichoic acid was digested with bacterial alkaline phosphatase at pH 9.5 for 18 hours at 37°C to release terminal phosphate groups. The ratios of total phosphorus to labile phosphorus from two determinations are given in Table 7. Labile phosphorus was also measured for untreated purified teichoic acid and was not detectable.

These results confirmed the prediction made by gel filtration that LS teichoic acid at about 70 glycerol phosphate units is, on the average, larger than GS teichoic acid at 38 units.

With these basic characterizations accomplished, it was necessary to confirm the polyglycerol phosphate backbone structure by showing that a one-to-one ratio of glycerol and phosphate existed.

Figure 4. Sephadex G-100 Chromatography of Purified Teichoic Acid.

Purified teichoic acids were filtered through 160 ml of Sephadex G-100. Deionized water was used for elution. Total phosphate was measured for each fraction and plotted versus K values computed for the corresponding fractions.

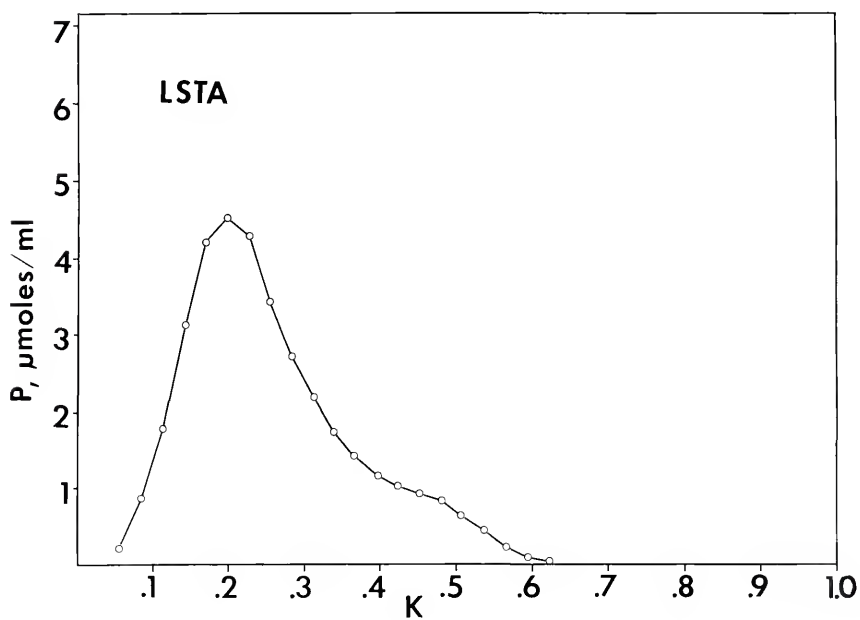
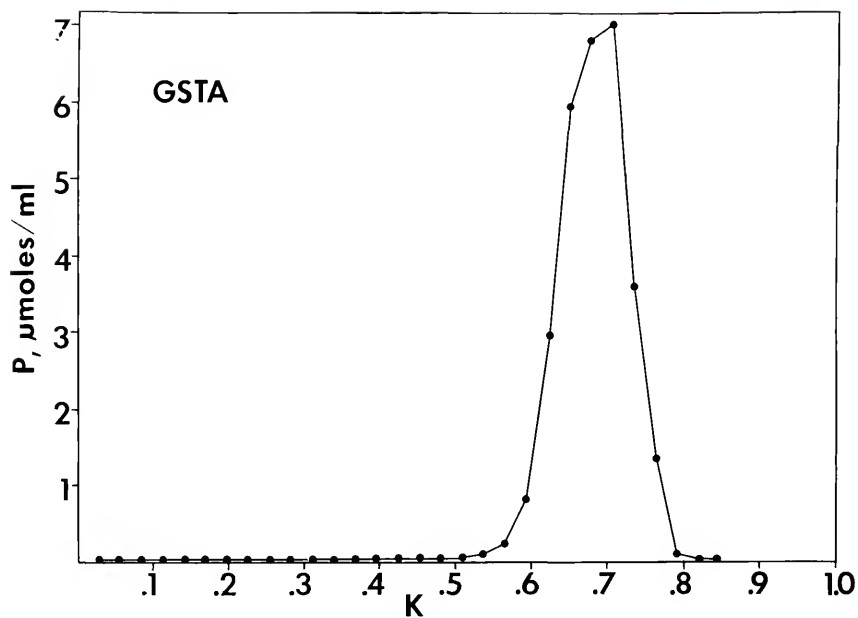


TABLE 7

Molar ratios of labile and total phosphate in
alkaline phosphatase digested, purified teichoic
acids.^a

Sample	Experiment number	Total: labile phosphate ^b
Purified GSTA	1	38
	2	38
Purified LSTA	1	66
	2	74

- (a) Teichoic acids were digested with bacterial alkaline phosphatase. After digestion inorganic and total phosphate were determined.
- (b) Values were determined by: Molar concentration of total phosphate/molar concentration of inorganic phosphate in digestion mixture.

Molar Ratios of Glycerol and Phosphate in
Purified Teichoic Acids

Purified teichoic acids were acid hydrolyzed to break down the glycerol phosphate backbone into glycerol mono- and di-phosphates, free glycerol, inorganic phosphate, and free amino sugars. The mixture was then treated with alkaline phosphatase. This mixture was assayed for free glycerol, inorganic and total phosphate. The molar ratios of glycerol to inorganic phosphate for two determinations are shown in Table 8. Analysis of total phosphate in these digested samples showed that alkaline phosphatase released 99% for GS and 87% for LS of the total bound phosphate.

With this preliminary confirmation of the poly (glycerol phosphate) nature of the backbone it remained to be proven that the amino sugars present were, in fact, covalently attached to the polyglycerol phosphate. This was accomplished by alkaline hydrolysis which breaks phosphodiester linkages but not glycosidic linkages. The general procedure used was similar to that of Van de Rijn and Bleiweis (83).

Characterizations of Alkaline Hydrolysates

Purified teichoic acid (GSTA only) was base hydrolyzed under conditions such as to break phosphodiester linkages without destroying glycosidic bonds. After neutralization, this hydrolysate was loaded onto a DEAE column to separate anionic compounds from cationic and neutral compounds. After the latter compounds were washed from

TABLE 8
Molar ratios of glycerol to inorganic phosphate.^a

Sample	Molar ratio (glycerol/P _i) ^b	
	<u>Lyophilized</u> ^c	<u>Dessicated</u> ^d
GS	0.86	0.86
LS	0.81	0.85

- (a) After acid hydrolysis and acid removal, teichoic acids were digested with alkaline phosphatase and free glycerol and inorganic phosphate were then determined.
- (b) determined by dividing the molar concentration of glycerol in the digest mix by the molar concentration of inorganic phosphate.
- (c) Determination where acid was removed by repeated (3 times) lyophilization and resuspension in small amounts of deionized water.
- (d) determination where acid was removed by vacuum evaporation.

the column, the anionic compounds were eluted using a linear salt gradient. The profile of this elution is shown in Figure 5. The total phosphate represented in this profile was 58% (19 μ moles) of the total phosphate (32.6 μ moles) that was loaded on the column. The total amino sugar recovery from the elution was 87% (7.9 μ moles) of the total amino sugar (9.1 μ moles) that was loaded on the column. The ratio of total amino sugar to phosphate in the single peak of the total amino sugar was about 3.2 for ten fractions with the most amino sugar.

The wash fraction contained the other 42% of the phosphate and 12% of the amino sugar. This fraction was further characterized by running on a Sephadex G-50 column. This profile is shown in Figure 6. The phosphate containing fractions (#36 - #61) were pooled and lyophilized. More than 99% of the phosphate and the amino sugar was recovered from the gel filtration column. The lyophilized sample was diluted to 0.1 M NaCl and applied to a CM-Sephadex column in order to separate cationic molecules from neutral ones. After washing, the sample was eluted with a salt gradient and the profile is shown in Figure 7. Phosphate was also measured but none was detected (<10 nmoles/ml). Amino sugar was, however, 100% recovered.

The two amino sugar peaks, the pooled wash, and a fraction midway between the two amino sugar peaks were analyzed for glycerol. The pooled wash and the midway fraction contained no detectable glycerol. The smaller of the amino sugar peaks (fractions #4 and #5) contained one glycerol for every 2.9 amino sugar residues while the larger peak (fractions #41 and #42) contained one glycerol for every 6.5 amino sugar residues.

Figure 5. Alkali-Hydrolyzed Teichoic Acid Elution on DEAE-Sephadex.

Alkali-hydrolyzed GSTA was applied to 9 g of DEAE-Sephadex. Nonadherent compounds were removed with a 500 ml wash of 0.1 M NaCl. Adherent compounds were eluted with a linear 0.1 M to 1.0 M NaCl gradient. Fractions were assayed for total phosphate and total amino sugars.

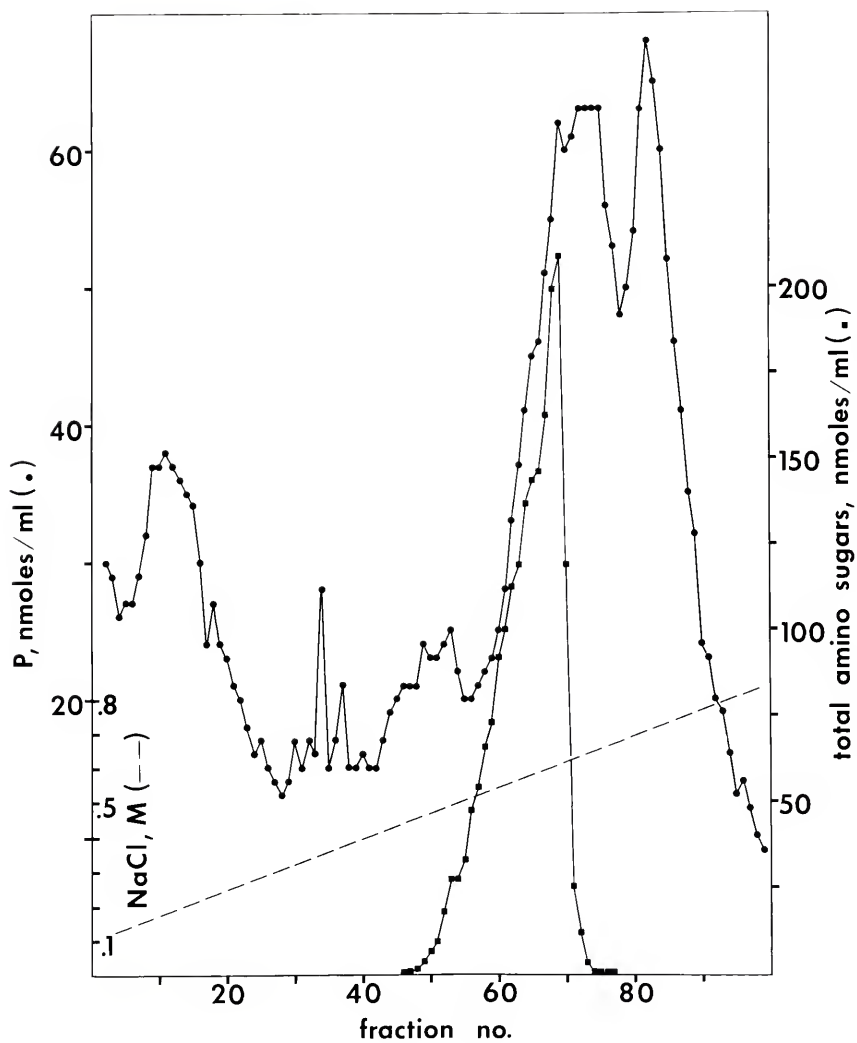


Figure 6. Sephadex G-50 Chromatography of Neutral and Cationic Components of Alkali-hydrolyzed GSTA.

The wash fraction from DEAE chromatography of alkali-hydrolyzed GSTA was run on 124 ml of Sephadex G-50. Deionized water was used for elution. Fractions were assayed for total (P_t) and inorganic (P_i) phosphate, and total amino sugars.

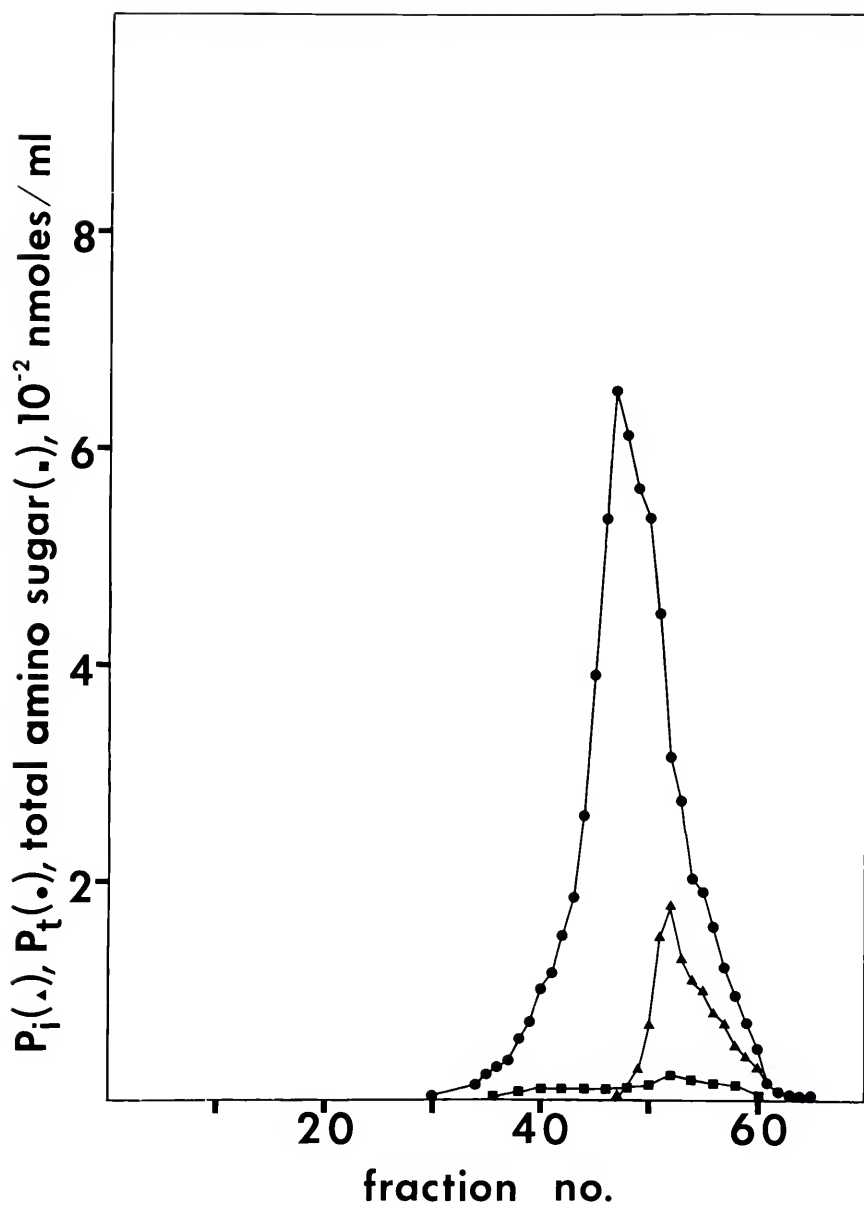
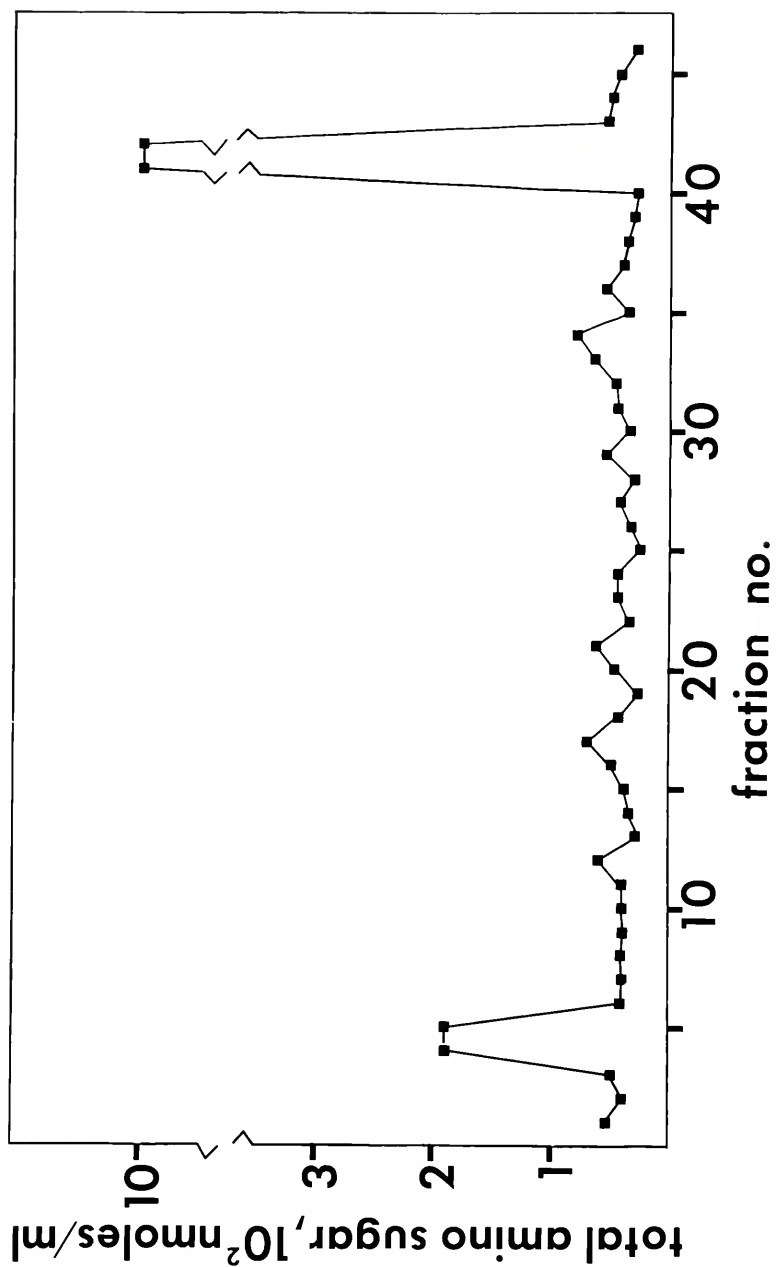


Figure 7. CM-Sephadex Chromatography of Neutral and Cationic Components of Alkali-hydrolyzed GSTA.

The phosphate-containing fractions from gel filtration were diluted to 0.1 M NaCl and applied to 1.9 g of CM-Sephadex. Neutral compounds were washed out by 50 ml of 0.1 M NaCl. A linear 250 ml 0.1 M NaCl gradient was used for elution. Fractions were assayed for total amino sugars.



DISCUSSION

Peptidoglycan Composition

Amino acid and amino sugar analyses of purified wall hydrolysates first suggested the presence of wall associated polymers other than peptidoglycan in the wall of *Arthrobacter crystallopoietes*. The boiling SDS extraction used to purify walls is a very rigorous method which should remove all noncovalently associated cellular materials. Despite this, small amounts of the amino acids aspartic acid, threonine, glycine, and valine were detected (Table 2). These amino acids are not usually found in the pentapeptide of peptidoglycan. Also, there was more glucosamine than could be accounted for by peptidoglycan structure and there was galactosamine present which has not been reported to occur as part of the peptidoglycan.

Krulwich et al. (55) have also reported on the amino acid and amino sugar composition of walls purified from *A. crystallopoietes* 15481. They also found small amounts of aspartic acid (0.15 mole/mole relative to glutamic acid in spheres, none found in rods) and glycine (0.14 mole/mole in spheres, 0.04-0.05 mole/mole in rods). They did not find valine or threonine but did find serine (0.13 mole/mole in spheres, none in rods). It is conceivable that serine and threonine might be confused in some amino acid analyzer programs since these amino acids are similar in structure. On the program used for my experiments, serine and threonine did elute next to each other but they were

distinctly different peaks on standard runs. Krulwich et al. (56) used *Chalaropsis* B enzyme (N,O-diacetylmuramidase) (41), lysostaphin (a mixture containing endo-N-acetylglucosaminidase and a peptidase which releases N-terminal alanine and glycine from cell walls of *Staphylococcus aureus* strain Copenhagen, hydrolyzes polyglycine cross-bridges, and, to a lesser extent, N-acetylmuramyl-L-alanine linkages) (15), and AL-1 (a peptidase which breaks peptide crossbridges and N-acetylmuramyl-L-alanine linkages) (79) in separate experiments to obtain soluble fragments which were separated by ECTEOLA cellulose and gel filtration. N-terminal and C-terminal amino acids were then determined for various fragments. By these analyses, they came to the conclusion that aspartic acid and serine were not connected with either peptidoglycan or teichoic acid. They suggested that these amino acids were either contaminants or part of a small additional peptide in the wall. According to their results, the glycine present was a part of some of the crossbridges in spherical populations. The glycine present in rod populations was not, however, part of the peptidoglycan and presumably could also be a contaminant or part of an additional peptide. Tipper et al. (79) working in the same lab as Krulwich et al. also have reported on the cell wall composition of spherical *A. crystallopoietes*. The composition they obtained is very similar to that of Krulwich et al. except that Tipper et al. reported about 0.03 μ mole/mg of wall of aspartic acid, serine, threonine, leucine, histidine, and methionine. Since their walls contained 0.42 μ moles of total glutamic acid/mg of wall, 0.03 μ mole/mg of wall would be 0.07 moles/mole of glutamic acid. The nature of these trace

amino acids and the ones which I have found is unclear but their presence calls for more research dealing with this problem.

Krulwich et al. (55) found additional glucosamine (spheres, 2.09 mole/mole of glutamic acid; rods, 1.74-2.01 mole/mole), galactosamine (spheres, 0.31 mole/mole; rods, 0.25-0.30 mole/mole), unidentified phenol-sulfuric acid-reactive material (spheres, 1.95 mole/mole; rods 1.04-1.37 mole/mole), and phosphate (spheres, 2.02 mole/mole; rods, 1.88-2.12 mole/mole). Tipper et al. (41) also reported similar results for glucosamine and galactosamine.

The walls analyzed in our laboratory also showed extra glucosamine (Table 1), although the amount was generally less than that found by Krulwich et al. (55) or Tipper et al. (70). Quantitative differences in accessory polymers between the present data and past data are to be expected since, (1) the media used differ and, (2) the strains of bacteria used could be different because of extensive subculturing. Galactosamine was also found by our lab but it was not quantitated in this experiment since at the time the identity of that peak was unknown. Phenol-sulfuric acid-reactive material was also found in TCA extractable material but it was found that this material could be separated from teichoic acid by chromatography on DEAE-Sephadex. It seems likely that this material may contain the glucose which showed up in paper chromatography of acid hydrolysates of TCA extractable material.

Krulwich et al. (55) were able to extract all the phosphate present in walls with 100% TCA at 60°C in less than one hour for both sphere and rod wall preparations. They concluded that this phosphate must be contained in teichoic acid since teichoic acids are extractable under those conditions. There are, however, other

accessory polymers which have been discovered since the paper of Krulwich et al. (55) and which are extractable under conditions milder than those of Krulwich et al. (5,8,51,65). Tipper et al. (79) also found a phosphate polymer which co-eluted with some of the polysaccharide solubilized by *Mycobacter* AL-1 enzyme. Nevertheless, Krulwich et al. have turned out to be correct in their assumption even though today their data would not be considered sufficient to establish the presence of a teichoic acid as opposed to a sugar 1-phosphate polymer or a phosphorylated polysaccharide. Krulwich probably realized this later because in a paper a year after the one described above, Krulwich and Ensign (53) refer to the same moiety only as a "phosphate-containing polymer of the cell walls".

Krulwich et al. (55) were correct about the lack of amino acid substitution of the teichoic acid. They found amino acids in their TCA extract but they were in the same ratios as were found in the peptidoglycan. They also incubated their purified cell wall in two alkaline buffers (pH 9.2 and pH 10.5) and found that no amino acids were released. This indicates that no amino acids are ester-linked to either polyols or to polysaccharides. In this work amino acid and amino sugar analyses of crude teichoic acids showed almost no amino acids present at all. The trace of alanine present could account for no more than one residue for every 100 glycerol phosphate residues in either GS or LS teichoic acid. It is more likely that this alanine arose from peptidoglycan contamination.

Experimental data (see Table 2) suggest that all four non-pentapeptide amino acids can be removed if the right protease is used. This confirms the hypothesis that these amino acids are not part of

the pentapeptide. This leaves the possibilities that these amino acids are contaminants, part of normal crossbridges, or part of a peptide attached to peptidoglycan.

Trypsin is known to be a specific endopeptidase which breaks peptide bonds in which the carbonyl group is donated by lysine or arginine. Most peptidoglycan basic structures are notoriously resistant to trypsin (74), even those which contain penultimate L-lysine in the tetrapeptide (64). The reason for this resistance is not clear but certainly those L-lysine residues which are involved in crossbridges would not be susceptible due to a lack of positive charge on the ϵ -amino group which is a necessary condition for trypsin digestion (49). My data indicate that trypsin has very little if any effect on the level of alanine in purified cell walls. The alanine ratios remain fairly constant regardless of which proteolytic enzyme is used. The alanine ratios reported here agree fairly well with those of Krulwich et al. (55). The alanine in excess of the two moles in the tetrapeptide probably is due to the L-alanine crossbridges proposed by Krulwich et al. (55). By C-terminal analysis, they estimated that from 60 to 70% of the peptides are crosslinked, which also agrees with the amount of excess alanine reported here.

If the effectiveness of the different proteolytic enzymes is compared, trypsin seems to be the most effective in removing the trace amino acids. The fact that trypsin removed most of these amino acids while α -chymotrypsin and pronase did not, may indicate that cleavage of a specific linkage to peptidoglycan is necessary to remove a small peptide. Also, it had earlier been noticed that if the concentration of pronase-digested wall to be analyzed was

increased tenfold, a broad spectrum of non-peptidoglycan amino acids appeared. This would seem to indicate that the trace amino acid represent only the most abundant amino acids present and, therefore, are the only ones normally detected since the levels of these non-peptidoglycan amino acids were so low. Whether or not trypsin can remove or lower the amounts of these other amino acids is unknown.

The possibility that the trace amino acids are no more than contaminants from membrane protein can not be discounted. This problem warrants further research. It is possible that by using a N-acetylmuramidase to solubilize the peptidoglycan, these amino acids could be characterized as belonging either to a high or low molecular weight fraction on gel filtration.

Perhaps the most important fact in this experiment is that extra glucosamine and galactosamine were present. Combined with the presence of significant amounts of phosphate, this suggested the possibility that a teichoic acid was present. Since Krulwich et al. (55) had found that phosphate could be TCA extracted, a similar kinetic experiment was carried out but using the milder temperature (4°C as compared to 60°C that Krulwich et al. (55) used) normally used to extract teichoic acid with the least degradation (8,51).

TCA Extraction

As was shown in the Results section, the initial rate of extraction of phosphate was relatively high and was almost the same for GS and LS. By only 8 hours, about 73% of the total extractable phosphate had already been solubilized. These initially high rates of extraction

then dropped dramatically so that another 67 hours were required to remove the remaining 27% of the extractable phosphate. Thus, the average rate of extraction for the first 8 hours was about 9% of the extractable phosphate per hour whereas the average rate after 8 hours was about 0.4% per hour. This amounted to more than a 22-fold difference. The explanation for this difference in rates is not known but it does seem to indicate that there may be two different types of reactions occurring. After 75 hours of extraction, 81% of the GS and 53% of the LS total phosphate was solubilized. If the slow rate of extraction remained constant, it would take 5 days for GS and 8 days for LS phosphate to be completely extracted. Ghuyssen et al. (34) reported that it took 3 weeks to extract more than 95% of the teichoic acid of *Staphylococcus aureus*. In the light of that study, it is likely that the rate of extraction does not remain constant but decreases further still with time.

The actual dry weight of teichoic acid recovered after 96 hours of extraction varied depending on the method used for recovery of teichoic acid from TCA supernatants. If the weights recovered are corrected for the percentage recovery, then the actual weight of teichoic acid in the native wall (GS or LS) may be as high as about 43% (crude TA dry weight percentage of purified wall dry weight). A small proportion of this crude teichoic acid is contaminating polysaccharide, so the actual figure is probably closer to around 40%. The amounts found in certain other gram positive bacteria range from 20% to 50% of the dry weight of the wall (8).

Characterization of Acid Hydrolysates

To determine whether or not this phosphate-containing polymer was a teichoic acid, acid hydrolyses were carried out under conditions sufficiently rigorous to degrade a glycerol or ribitol teichoic acid to its monomers (3).

Table 3 shows the phosphate-containing monomers of acid-hydrolyzed crude teichoic acid. The ascending technique seems to give the least ambiguous results. The hydrolyzed teichoic acid spots matched fairly well with the mobility of 1,2 diphosphoglycerol. The fact that there were two spots in both the standards and the hydrolysates is probably due to some inorganic phosphate which should migrate a little farther than 1,2 diphosphoglycerol (50) and exists as a minor contamination in the 1,2 diphosphoglycerol standard. Hydrolyzed cardiolipin also showed a pattern of light and dark spots because this compound upon hydrolysis should give rise to 1,3 diphosphoglycerol (R_f , 0.12) and glycerol monophosphates (R_f , 0.35). These mobilities agree well with those found in the literature (7,29,50). The hydrolyzed teichoic acid spots on the descending run make it more difficult to choose between the 1,2 and the 1,3 compound. Indeed, the hydrolyzed teichoic acid samples may be a mixture of both which runs together on paper chromatography. This is actually what one would expect since a certain amount of ester migration can occur under acidic conditions (3). It is noteworthy that no glycerol monophosphates were detected but the reason for this is not clear.

In Table 4, it seems quite clear that the mobility of the hydrolyzed teichoic acid spots matched that of glycerol. The true picture,

however, is somewhat more cloudy than that. Anhydroribitol is reported to have the same mobility as glycerol under the conditions of chromatography used in this experiment. Apparently, the conditions of hydrolysis used for both the samples and the standard (2 N HCl, 100°C, 3 hours) were not sufficient to convert a detectable amount of ribitol to anhydroribitol. Anhydroribitol is reported to give a slow reaction with periodate-Schiff reagent (7) and so the fast reaction given by the hydrolyzed teichoic acid samples confirms that glycerol, not anhydroribitol, was present. The yellow spots which did not migrate were probably sugars which are characterized in Table 4.

Both GS and LS crude teichoic acids appear to have the same sugars present as evidenced by the banding patterns obtained in the ethyl acetate-pyridine-water solvent system. Both preparations had a silver nitrate reactive spot which migrated the same distance as glucose. Both preparations also had darker areas in the long tails which extended from the origin to about halfway to the solvent front. These darker areas corresponded well to glucosamine and galactosamine standards. Later analyses confirmed the covalent linkage of these latter sugars to teichoic acid. The glucose is not connected with teichoic acid but may be part of the wall nevertheless.

Since the spots that were presumably glucose did not exactly correspond in mobility to glucose in every run, glucose was added as an internal standard to the GS and LS acid-hydrolyzed preparations (Table 6). In this chromatograph standard glucose and sample glucose

migrated as a single spot. These spots moved slower than the glucose standard alone. The probable cause for this is the presence of small amounts of salt in the sample.

Amino Acid and Amino Sugar Analyses of Teichoic Acid

Amino acid and amino sugar analyses confirmed the identity of the glucosamine and galactosamine indicated by paper chromatography. These analyses also indicated that there was about five times as much glucosamine present as galactosamine.

The traces of alanine present would contribute less than one residue for every 100 glycerol phosphate residues. For this reason, it is felt that this alanine probably arose from contamination by peptidoglycan.

DEAE Chromatography

To purify the crude teichoic acids, ion exchange chromatography was used. The teichoic acid preparations were applied to the DEAE-Sephadex under conditions which allowed the anionic teichoic acids to stick to the resin. Water washes removed a similar amount of phenol-sulfuric acid reactive material from both preparations. This material is presumed to contain the glucose present in crude teichoic acid samples as demonstrated by paper chromatography. Lyophilization of this material rendered it water-insoluble and so no further analyses of this material were carried out. No phenol-H₂SO₄ reactive material

was found in the salt-eluted fractions, so glucose is presumed not to be part of the teichoic acid structure.

The profiles of Figure 3 show that GSTA and LSTA are different in their affinity for DEAE groups. The peaks of phosphate eluted at salt concentrations which were about 0.1 M different. This suggests that there may be a difference in net charge of the molecules being separated. These profiles also show that the amino sugar curve closely matched that of phosphate. If the ratios between phosphate and amino sugar are calculated for each fraction in the main peaks, these ratios are relatively constant. They are so constant that in more than 95% of the cases, the differences can be attributed to a change from the mean ratio of an amount that can be accounted for by a change of one or two residues. Thus suggests that these two moieties were associated with each other and not simply eluting close to one another.

Dry weight recoveries were quite low as compared to phosphate recoveries. This is probably due more to inaccuracies in dry weight measurement (resulting from incomplete water removal from samples) than to a removal of contamination.

Acid Hydrolyses of Purified Teichoic Acids

The amount of amino sugar in the two preparations was different but not radically so. The mild acid hydrolysis only demonstrated that about one half of the amino sugars were N-acetylated, but this is strictly a minimum figure. Even under these mild conditions some hydrolysis of the N-acetyl groups probably occurs. It is therefore

probable that most amino amino sugar was N-acetylated. DMAB reagent showed a limited reactivity to amino sugar associated with teichoic acid because the N-acetyl amino sugar is bound at the C-1 position. N-Acetylation with acetic anhydride did not increase this reactivity. This would seem to indicate that all the amino sugars are already N-acetylated. When the teichoic acid was acid hydrolysed a large increase in reactivity to DMAB occurred due to exposure of C-1.

Gel Filtration

Teichoic acids were also characterized by gel filtration. It was found necessary to use Sephadex G-100, a gel with a molecular weight exclusion limit much higher than any known teichoic acid molecular weight. This is probably due to the high charge and extended shape common to teichoic acids (25,27). The profiles in Figure 4 again show that there was a considerable difference between GSTA and LSTA. The most obvious cause for this is a difference in molecular weight but if there were a large difference in charge or shape of the molecule, it could affect the retention of the teichoic acid molecules.

Determination of Chain Length

To further characterize the teichoic acids and to determine if molecular weight was the determining factor in the gel filtration profile, chain lengths were determined. Average chain length can be determined by releasing the terminal phosphomonoester group

with alkaline phosphatase and measuring the inorganic phosphate released and comparing that to the total phosphate content. This gives the average number of glycerol phosphate residues per chain if there is a one-to-one correspondence of glycerol to phosphate. These determinations showed that GSTA at 38 glycerol phosphate units was only about half as long as LSTA at an average of 70 units. These numbers are representative of purified teichoic acid preparations only and may not reflect accurately the native chain lengths as found in the wall (8). TCA is known to break phosphodiester bonds (77). Phosphodiester bonds not only link the glycerol phosphate monomers, they are also responsible for linking the teichoic acid to peptidoglycan in the only well-characterized cases to date (22,38,77). It is not surprising then that a certain amount of degradation of chain length would occur upon TCA extraction of teichoic acids. Indeed, this has been observed in several cases (8,34,51). The kinetic data seem to indicate that there may be two mechanisms of extraction. This may be reflective of the phosphodiester links which bond the backbone units and those which may link the backbone to peptidoglycan. Even so, the difference between GSTA and LSTA would be hard to explain by differing labilities to acid extraction when their composition is so similar. Thus, even if the chain lengths do not accurately reflect those in the native wall, there still should be a significant difference between the lengths of GSTA and LSTA in the wall.

It is very probable that the differing chain lengths of GSTA and LSTA account for some of the differences observed in ion-exchange chromatography. DEAE chromatography has been shown to separate oligonucleotides according to net negative charge which is a function

of chain length under conditions minimizing charge and secondary binding forces of the purine and pyrimidine bases (72,81,85). It is reasonable to assume then that LSTA with an average of 70 negative charges per molecule is going to be eluted at a higher ionic strength than is GSTA which only has an average of 38 negative charges per molecule. This is, in fact, what is observed (Figure 3). This reasoning can be extended to the material which elutes at lower ionic strengths which would be of lower chain length than the bulk of the material. This material probably resulted from degradation by TCA hydrolysis of backbone phosphodiester bonds.

The difference in chain length also is reflected in the gel filtration profile but it should be noted that the difference in charge per molecule may also play a role in the behavior of teichoic acids on Sephadex G-100. Pharmacia states that Sephadex G-100 contains small numbers of carboxyl groups and, therefore, at low ionic strengths, negatively charged molecules may be excluded from the gel to a greater extent than an uncharged molecule of the same molecular weight (67).

Glycerol-Phosphate Ratios

Many of the above conclusions can only be drawn by assuming that there is a poly (glycerol phosphate) backbone. To confirm the presence of such a backbone, it is necessary to show a one-to-one ratio between glycerol and phosphate. As Table 8 shows, this is the case for both GSTA and LSTA.

Alkaline Hydrolysis

The final characterization of teichoic acid was by alkaline hydrolysis. The primary goal of this experiment was to establish whether or not the amino sugars were covalently attached to teichoic acid. Alkaline hydrolysis leaves glycosidic linkages intact while cleaving phosphodiester linkages where free hydroxyl groups are available on neighboring carbons allowing for a cyclic phosphate intermediate to form (50).

Figure 5 shows that a substantial amount of amino sugar elutes from DEAE-Sephadex as a single peak. Since only anionic molecules should be bound to this resin, this suggests that the amino sugar is still bound to glycerol phosphate. This peak accounts for 87% of the amino sugar in the alkali-hydrolyzed GSTA. Thus, the majority of amino sugar is covalently attached to teichoic acid and it suggests that the poly (glycerol phosphate) backbone is 2,3 linked rather than 1,3 linked. In a 1,3 teichoic acid if a glycosidic bond ties up C-2 of a glycerol phosphate residue, then there is no free hydroxyl on that residue for the necessary cyclic phosphate to form. For this reason, one expects only glycosylglycerol for substituted residues. In contrast, glycosylglyceromonophosphates can only result from a 2,3-linked teichoic acid structure. It has never been found in membrane teichoic acids and only in a few cases in wall teichoic acids (32,63). The amount of amino sugar exceeds the amount of phosphate in the amino sugar peak by a factor of about 3.2. This suggests that there may be an average of three amino sugars linked to each other by

glycosidic bonds and this trimer is attached to a single glycerol phosphate residue.

The other 12% of the amino sugar in the alkaline-hydrolyzed GSTA was characterized as being low molecular weight because it was almost completely included in a Sephadex G-50 gel (Figure 6). This material was then eluted on a cation exchange resin. All the remaining phosphate was washed through the column with 0.1 M NaCl. Two peaks of amino sugar were then eluted using a linear NaCl gradient. Together these accounted for all of the amino sugar from the DEAE wash fraction. No phosphate was detected in either fraction. Both peaks did contain glycerol. The smaller peak contained about three amino sugar residues for each glycerol residue while the larger peak contained 6.5 amino sugars per glycerol. It would seem, therefore, that CM-Sephadex acts in manner analogous to DEAE-Sephadex in that it can separate positively charged molecules with similar charge densities by chain length. These data also suggest that amino sugar is covalently attached to teichoic acid and may exist as trimers or hexamers attached to single glycerol residues. The significance of the larger amount of the hexamer in this relatively small fraction of amino sugar is not known. Although these results were obtained only for GSTA, it seems reasonable that LSTA follows a similar but not identical pattern.

Summary of Teichoic Acid Structure

Arthrobacter crystallopoietes contains a glycerol wall teichoic acid in both sphere and rod morphologies in approximately equal amounts. This teichoic acid is 38 glycerol phosphate units long in spheres and about 70 units long in rods. N-Acetylhexosamine (N-acetylglucosamine, about 83% and N-acetylgalactosamine, about 17%) is attached by a glycosidic linkage from C1 of the hexosamine to C1 of glycerol. On the average, there are 11 hexosamines per chain in sphere teichoic acid while there are about 15 hexosamines per chain in the rod teichoic acid. These hexosamines may exist in polymerized side chains which have three sugars on the average. Thus, only about 11% (GS) or 7% (LS) of the glycerol phosphate units are substituted. The poly (glycerol phosphate) backbone is 2,3 linked for GSTA and probably for LSTA also. This is the first teichoic acid that has been characterized to this extent in the genus *Arthrobacter*.

Possible Roles for Teichoic Acids in *Arthrobacter*

One role of teichoic acids that is well established is binding of cations, particularly divalent cations (6,10,26,42,46). Evidence has been presented that wall and membrane teichoic acids provide a controlled reservoir of bound Mg^{+2} for membrane bound enzymes (46) which require it for maximal activity (2,21,66). It has been suggested that the wall teichoic acid scavenges Mg^{+2} from the environment and transfers it to lipoteichoic acid which interacts with the membrane

enzymes. Baddiley et al. (10) have shown that alanylated teichoic acid interacts less strongly with Mg^{+2} and, therefore, the degree of D-alanine substitution may act as a controlling influence on Mg^{+2} binding.

It seems reasonable that the teichoic acid in *A. crystallopoietes* could serve the same function since its structure is similar to some of those used in the above mentioned studies. If this were true, then it could be that the longer chains found in rod shaped bacteria might more effectively scavenge Mg^{+2} than the short chains of the spheres. This would be consistent with the higher growth rate of rods which would presumably necessitate a greater flux of Mg^{+2} to the membrane.

Another function of teichoic acids is suggested by the studies of Burge et al. (20) and Milward and Reavely (62). By examination of peptidoglycans containing teichoic acid and those which had teichoic acid artificially removed, these authors found that extraction of teichoic acid affects the flexibility of the peptidoglycan. It was suggested (20) that since teichoic acid can pass through the pores in the outer layers of peptidoglycan it would resist shear between adjacent peptidoglycan sheets and still allow for expansion or contraction of the wall.

The most interesting function as far as this work is concerned is suggested by several studies in which teichoic acid changes effect autolytic enzymes.

Holtje and Tomasz (45,80) have described an N-acetylmuramyl-L-alanine amidase of *Pneumococcus* which required choline residues in the wall teichoic acid for maximal binding. If ethanolamine residues were

substituted, the cell walls were resistant to the autolysin. Besides lowered binding of the autolysin to the wall, this effect was due to the inability of the altered teichoic acid to convert the autolysin to its active form. Herbold and Glaser (43) have described a similar N-acetylmuramyl-L-alanine amidase in *Bacillus subtilis*. Again cell walls lacking teichoic acid had a much lower affinity for the enzyme than those with normal levels of teichoic acid. In a different strain of *Bacillus subtilis*, Brown et al. (16,18) had such trouble purifying autolysin from teichoic acid that they suggested that the two molecules might be covalently attached to each other. They speculated that the close association between autolysin and the teichoic acid might serve to localize the autolysin for maximum utilization of substrate.

In a temperature conditional morphological mutant of *Bacillus subtilis*, Boylan et al. (12,13,24) found that at the non-permissive temperature teichoic acid was deficient. These mutants grew as normal rods at 30°C but changed to irregular spheres at 45°C. The walls of the spheres were also deficient in N-acyl muramyl-L-alanine amidase. Rogers et al. have found similar results in seven rod A mutants of *Bacillus subtilis* (17,71) and *Bacillus licheniformis* (33).

Other authors (44,75) have also suggested that autolysins may play a significant role in the determination of morphology in all bacteria.

In *A. crystallopoietes* it has been shown that the activity of N-acetylmuramidase correlates with morphogenesis (53). The high activity of the autolysin in spheres results in the relatively short glycan backbones found in the peptidoglycan. In the same way, the low activity of this enzyme in rod walls results in relatively

long glycans. The length of the glycan chains probably influences the rigidity of the peptidoglycan and, therefore, the morphology of the organism (55).

The present work has shown that there is almost a two-fold difference in the length of teichoic acids isolated from spherical and rod-shaped *A. crystallopoietes*. In light of the correlations between morphology, autolysins, and teichoic acids discussed above, it does not seem unreasonable that this difference may play a role in morphogenesis.

Phosphate analyses of purified wall show that there is slightly more phosphate per mg of rod wall than in sphere wall (rods, 21 $\mu\text{g}/\text{mg}$ wall; spheres, 18 $\mu\text{g}/\text{mg}$ wall). Since the rod teichoic acid has almost twice the phosphate per chain as the sphere teichoic acid because of its length, this means that there are about one half as many rod teichoic acid chains as there are sphere teichoic acid chains. If autolysin were binding to the teichoic acid chains, the rod wall would present about one half as many binding sites proximal to the wall as would the sphere wall.

A question that arises from this model is why the autolysin does not cleave the N-acetylmuramyl-N-acetylglucosaminyl residue in peptidoglycan to which it is attached. This would be disadvantageous to the cell because both teichoic acid and autolysin would be lost from the cell which represents a considerable energy investment wasted. One possible solution to this problem could lie in the specificity of the autolysin. The autolysin in *A. crystallopoietes* thought to be responsible for control of glycan length is an N-acetylmuramidase. It is possible that this enzyme can not hydrolyze at the reducing

end of an N-acetylmuramic acid which has the C-6 hydroxyl tied up in covalent linkage. Lysozyme is known to be inhibited under these conditions and teichoic acids are known to inhibit lysozyme in certain cases (78). The only well characterized linkages of teichoic acid to peptidoglycan are to the C-6 hydroxyl of N-acetylmuramic acid. In both our laboratory and that of Krulwich et al. (55), lysozyme was found to be poorly lytic against purified cell walls of *A. crystallopoietes*. *Chalaropsis* B enzyme, on the other hand, completely solubilizes these walls. *Chalaropsis* B enzyme is an N,O-diacetylmuramidase which is similar to lysozyme except that it will cleave at the reducing ends of N-acetylmuramic acids which are substituted in the C-6 position. So, apparently something is attached at this position in *A. crystallopoietes*. It is possible that this might be teichoic acid but it could also be polysaccharide. If it were teichoic acid, it may be the reason why autolysin does not remove teichoic acid. Krulwich and Ensign (53) did find that the autolysin preferentially solubilizes peptidoglycan which is low in phosphate content. Another reason for this result might be steric hindrance of the enzyme by the teichoic acid.

It may be that teichoic acid does not bind the autolysin at all but only serves in substrate recognition by the enzyme as suggested by Higgins and Shockman (44). In *Streptococcus faecalis*, the N-acetylmuramidase binds to TCA extracted walls but these walls are hydrolyzed slower by autolysin than untreated walls. The TCA extracted walls are, however, a better substrate for lysozyme.

It is also possible that the divalent cation binding property of teichoic acids is important in regulating the autolysin. Clearly, there is much more to be found out about the roles of teichoic acids in *A. crystallopoietes*. Hopefully, further research will shed light on these roles.

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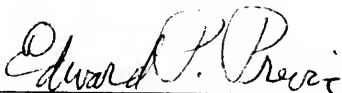
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BIOGRAPHICAL SKETCH

John H. Hellmuth was born September 3, 1952, in Jacksonville, Florida. There he attended The Bolles School and graduated in May, 1970. In December, 1973, he received the degree of Bachelor of Science with honors, with a major in biological sciences with an emphasis in Microbiology from North Carolina State. From January, 1974, until the present time, he has pursued his work toward the degree of Doctor of Philosophy in the Department of Microbiology and Cell Science, University of Florida.

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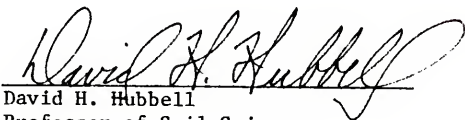
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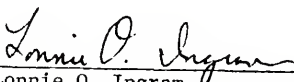
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Professor of Soil Science

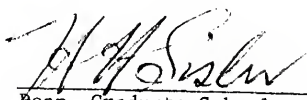
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